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Some Factors Affecting a Commercial Kit for Radioimmunoassay of Digoxin Using Tritiated Digoxin

By *A. B. T. J. Boink, H. H. Kruyswijk, A. F. Willebrands,* and *A. H. J. Maas*

From the Department of Cardiology, University Hospital, Utrecht, and the Department of Cardiology and Clinical Physiology, Wilhelmina Gasthuis, University of Amsterdam (The Netherlands)

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Summary: Some factors affecting results of digoxin determinations using one commercially available radioimmunoassay kit are described and discussed.

Serum of pregnant women, cord blood, amniotic fluid and serum of patients taking spironolactone may show erroneously high digoxin activity due to lack of specificity of the antiserum.

Cross-reaction with digitoxin was found to vary substantially with antibody-lot.

Haemaccel (5 g/l) in the sample leads to too low results. When ethanol (100 g/l) is present results are too high.

The need for testing the specifity of every new lot of antiserum before use is stressed.

Einen käuflichen Radioimmunoassay für Digoxin mit tritiiertem Digoxin beeinflussende Faktoren

Zusammenfassung: Es werden einige Faktoren, die die Ergebnisse von Digoxin-Bestimmungen mit einen käuflichen Radioimmunoassay-Besteck beeinflussen, beschrieben und diskutiert.

Aufgrund mangelnder Spezifität des Antiserums können Serum Schwangerer, Nabelschnurblut, Fruchtwasser und Serum von Patienten, die Spironolacton einnehmen, fälschlich zu hohe Digoxinwerte zeigen.

Die Kreuzreaktion mit Digitoxin variierte mit der Antikörper-Charge.

Haemaccel (5 g/l) in der Probe führt zu zu niedrigen Ergebnissen. In Gegenwart von Ethanol (100 g/l) sind die Ergebnisse zu hoch. Die Notwendigkeit, die Spezifität jeder neuen Antiserum-Charge vor Gebrauch zu testen, wird betont.

Introduction

The radioimmunoassay of digoxin in serum and plasma is now a fairly common laboratory procedure. The method and its clinical relevance have been extensively reported in the literature (1–4). Since the report of the generation of a specific antibody by *Butler* (5) and the introduction of a radioimmunoassay procedure by *Smith* (6) and *Evered* (7), a number of investigators have reported various factors affecting the results of this assay. Some of the most important factors are: quenching (8), radioactive contamination of patient's serum (9), chemiluminescence of serum (10), lack of specificity of the antibody used (11), low intrinsic association constant of the antibody-antigen complex (12), and effects of albumin on this complex (13).

In our laboratories the digoxin radioimmunoassay has now been in use for about four years. With regard to the above mentioned factors, we investigated the specificity of different batches of antiserum to digoxin, and to digitoxin and spironolactone. Moreover, measurements in biological fluids during pregnancy revealed interferences with those batches of antiserum which also showed a high degree of cross reaction with these drugs. In addition, false digoxin radioimmunoassay results also resulted from the presence of ethanol or a plasma expander in the assay tube.

Materials and Methods

Chemicals

Lanoxitest-β kit (Wellcome Reagents Ltd., Beckenham, U.K.), containing antibody, standard digoxin solution, tritiated digoxin, horse serum, buffer (phosphate-albumine buffer pH 7.40), and albumin coated activated charcoal.

Crystalline digitoxin (Ned. Ph. VII, Nogepha, Alkmaar, The Netherlands).

Crystalline spironolactone (Searle G. D. & Co., Chicago Ill. 60680).

Haemaccel (Farbwerke Hoechst A.G., Abt. Behring Präparate, Frankfurt a. M., Germany).

Macrodex and Rheomacrodex (Poviet Produkten B.V., Amsterdam, The Netherlands).

Scintillation liquid was either Instagel (Cat. no. 6002174 Packard Instrument Co., Inc., Downers Grove, Ill. 60515) or a toluene (1 liter)-Titron-X-100 (0.5 liter)-PPO (5 g)-POPOP (0.05 g) mixture.

Procedures

Digoxin radioimmunoassay is performed according to the supplier's instructions, and with the modifications reported earlier from our laboratories (11).

Solutions of digoxin, spironolactone, digitoxin, Haemaccel, Rheomacrodex and Macrodex are prepared in the buffer supplied with the test kit, and assayed in the presence of horse serum to compensate for differences in standards and samples.

As a measure of the intrinsic association constant of the antigen-antibody complex, the residual radioactivity after 30 minutes adsorption to activated charcoal is measured in the supernatant after centrifugation, and expressed in percentage of the activity after one minute adsorption.

For extraction, 10 ml methylene chloride is added to a 2 ml sample. After mixing thoroughly but gently for 15 minutes, the tubes are centrifuged, the upper layers removed, and 6 ml of the methylene chloride fraction evaporated to dryness. The residue is dissolved in 0.1 ml ethanol and made up to 1.0 ml with buffer.

Beta counting is conducted in either the Tricarb Model 2425 (Packard) or in the Mark II (Nuclear Chicago Corp., Des Plaines, Ill. 60018) liquid scintillation counter. Quench correction was made by the external standard ratio method.

Plasma or serum samples from patients on digoxin were drawn at least six hours after the last dose. Pools of maternal- and cord blood-serum and of amniotic fluid were obtained at delivery from nine women, who were not taking any medication.

Results and Discussion

After we started to determine plasma digoxin routinely in 1971, we were confronted with several types of interference with this assay.

Table 1 summarizes an in vitro comparison of the specificity of different antibody batches. The measurements were performed at least in duplicate in two laboratories. The given values are means of these measurements with a standard deviation of 0.2 µg/l. A control digoxin serum containing 3.0 µg/l (column e) was measured throughout to ensure the validity of the results.

Spironolactone

In some patients without clinical signs of digoxin intoxication high plasma digoxin concentrations, suggesting toxicity, were measured. Some of these patients were medicated with both digoxin and spironolactone.

So we studied another group of patients using spironolactone only. Apparent digoxin concentrations of 0.3–1.7 µg/l were found in seven patients receiving daily doses of spironolactone of either 100 or 150 mg (16). In table 1*a* the apparent digoxin concentrations, measured with different antibody charges at fixed spironolactone concentrations added in vitro are presented. Figure 1 shows the response of two antibody charges to increasing concentrations of spironolactone. Using antibody of charge no. K3650, an increasing cross reaction with spironolactone was found, but no significant effect was observed when antibody K7200 was used. So it is clear that the interference by spironolactone depends on the antibody charge used.

From the active metabolites of spironolactone we could study only the effect of canrenoate. This showed no cross reaction, probably because of opening of the γ-lactone ring. (See fig. 2). Unfortunately canrenon, the other metabolite which has a γ-lactone ring, was not available.

Tab. 1. Specificity and affinity characteristics of different antibody lots

	a	*b*	*c*			*d*	*e*
	500 µg/l spironolactone	20 µg/l digitoxin	pooled samples from pregnant women not on digoxin			amount of dissociation[e]	control digoxin serum (3µg/l)
antibody lot number			maternal serum	amniotic fluid	cord blood	$\frac{cpm_1 - cpm_{30}}{cpm_1} \times 100$ [%]	
	[µg/l]	[µg/l]	[µg/l]	[µg/l]	[µg/l]		[µg/l]
X[a]	1.4[b]	3.3	0.3	1.5	1.7	73	3.0
K3656	0.5	2.0	(0.2)	1.5	1.9	87	2.9
K5012	(0.0)	1.9		1.6			3.0
K6974	0.6	1.1		(0.25)		85	3.2
K7200	(0.0)	0.9	(0.1)		(0.2)	88	3.3
K8184	(0.2[c])	1.0[d]			(0.2)	70	2.9
K9100	0.3[c]	1.0[d]	(0.0)	(0.0)	(0.2)	65	3.1

Figures given are means of 4 to 8 determinations. Standard deviation: 0.2 µg/l
Figures between parentheses are not significant ($p > 0.05$)
a: lot number unknown: delivered January 1972
b: spironolactone concentration: 250 µg/l
c: spironolactone concentration: 2000 µg/l
d: digitoxin concentration: 40 µg/l
e: cpm_1 = after 1 minute time of adsorption to charcoal
cpm_{30} = after 30 minutes time of adsorption to charcoal

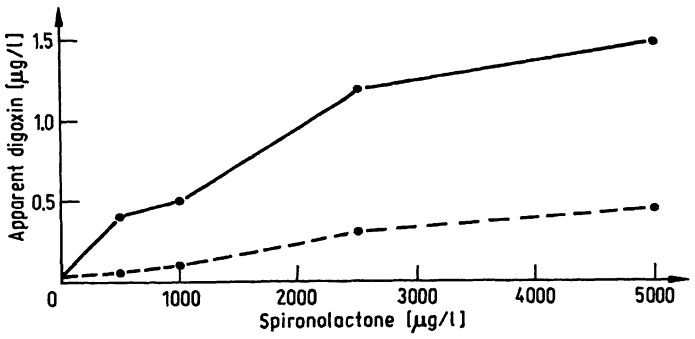

Fig. 1. Response to spironolactone of two different antibody charges used in the radioimmunoassay of digoxin.
●——● antiserum lot number K3650
●– –● antiserum lot number K7200
Each point represents the mean of four determinations. The standard deviation was 0.2 µg/l.

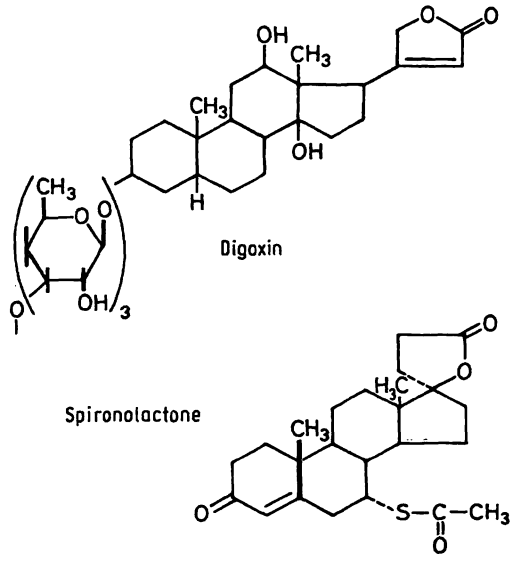

Fig. 2. Formulas of digoxin and spironolactone. The active metabolites of spironolactone are formed by splitting off of the thiolacetyl group – canrenon, which is in equilibrium with canrenoate by opening of the γ-lactone ring.

In an article of *Ravel* (18) negligible interference by spironolactone and prednisone is described. Unfortunately he did not mention the lot numbers of the kits he used, as his results using the Corning and Kallestadt kit suggest an antibody-dependent interference.

Moreover the dose of spironolactone administrated to the patients of *Zeegers* (11) was twice that received by the patients of *Ravel*.

Digitoxin

The manual of the digoxin radioimmunoassay kit states that this kit can also be used for the estimation of serum digitoxin: "digitoxin will crossreact in the assay with about 1/10th of the activity of digoxin on a weight basis". The first kits we used gave satisfactory results for the assay of digitoxin. However, when new lots were delivered, the cross reaction of digitoxin was reduced to about 1/20th, and even 1/40th of the activity of digoxin. Because of this lack of sensitivity to digitoxin we had to reject the kit for the digitoxin assay. To illustrate the effect of the quality of the antibody Table 1 *b* presents the apparent digoxin concentration in a serum containing 20 µg/l digitoxin, measured with different charges of antibody. One can conclude that a varying degree of cross reaction is found with different antibody charges. Therefore, when both substances are administered to patients, no uniform correction factor for the interference of digitoxin in the radioimmunoassay of digoxin can be applied as suggested by *Kuno-Sakai* (14). In the paper of *Kubasik* et al. (15), who compared five commercially available kits for the radioimmunoassay of digoxin, two different lot numbered kits of each supplier, showed only minor variations. In our study, the antibody of the low numbered lots shows much more cross reaction than the high numbered.

Pregnancy (maternal blood, amniotic fluid, and cord blood)

In an attempt to study the placental passage of digoxin we found apparently toxic digoxin concentrations up to 6.2 µg/l in amniotic fluid and cord blood during pregnancy in five women taking digoxin. Maternal serum digoxin concentrations were in the normal therapeutic range. None of the babies showed any sign of digitalis intoxication. The result of the assays suggested accumulation of digoxin in amniotic fluid and cord blood. However, values for digoxin in maternal plasma ranged from 0.2 to 0.7 µg/l in nine pregnant patients receiving no digoxin. Corresponding values for digoxin in amniotic fluid and cord blood plasma ranged from 1.35 to 2.5 µg/l (see also *Jambroes* et al. (16)). This interference was also found to depend on the antibody charge used. For this study pooled samples of maternal and fetal plasma, and amniotic fluid from the same pregnant women, not on digitalis, were assayed for apparent digoxin activity. The results are presented in table 1*c*, showing again an antibody charge-dependent interference with the radioimmunoassay of digoxin.

A study on transplacental passage of digoxin, using a reliable antibody, will be published elsewhere.

Influence of the association constant of the antibody

Zeegers et al. (11) observed that variations in the contact-time of the antigen-antibody system with charcoal (to remove unbound digoxin) can greatly influence the results of the tests. They concluded that this period should be meticulously standardized. *Smith & Haber* (12)

(12) ascribed this contact-time effect to differences in the intrinsic association constant of the antibody-antigen complex. To investigate this phenomenon we measured the residual antibody-bound [^{3}H]digoxin activity after 30 minutes contact with charcoal and expressed it as the percentage of the activity after 1 minute contact-time, using all the available batches of antibody. The apparent rates of dissociation of the antibody-antigen complex for the different batches are listed in table 1*d*, showing a variation from 65 up to 88 percent. Zero values ($\frac{CPM_o\text{-Blank}}{\text{Total CPM-Blank}}$) were between 50 and 60%. *Kuno-Sakai* et al. (17) who also studied the effect of variations in contact time to charcoal stated that the magnitude of this effect varies with the manufacturer. Our results however emphasize differences between antibody charges from one manufacturer.

Smith & Haber related a poor intrinsic association constant of the antibody-antigen complex with a lack of specificity of the antibody. From the results in Table 1*a–d*, especially with antibody charges no. x, K8184, and K9100, one can see that their conclusion does not hold for all batches; from the results with those that we studied, it can be concluded that cross reaction with one interfering substance is concomittant with interference by other crossreactants.

With reference to the problems described so far, we would like to quote *Th. W. Smith & E. Haber* in Pharmacological Reviews 1973 (page 224). "Unfortunately, commercial suppliers of radioimmunoassay material have in general supplied the purchaser with inadequate data characterizing the antibodies supplied. The responsibility for quality control therefore rests with the user. Minimum documentation of the suitability of a given antibody for radioimmunoassay use would require hapten inhibition studies to define specificity, and charcoal contact time studies to define the range of contact times which could be used without introducing an unacceptable degree of error". In this study we have followed these suggestions for radioimmunoassay kits obtained from one manufacturer. Differences between several commercially available digoxin radioimmunoassay kits are extensively reported in literature (15).

Haemaccel[1])

In a study on digoxin pharmacokinetics in patients during cardiopulmonary bypass we sometimes found very low or even apparently negative plasma digoxin concentrations (tab. 2). No corrections were made for background counts in these samples. As a control the pump priming was measured, which gave an apparently negative digoxin level. As no radioactive contamination could be detected all constituents of the pump priming were tested. From these, Haemaccel turned out to interfere negatively with the radioimmunoassay of digoxin. We decided to study this effect more comprehensively.

Tab. 2. Digoxin concentration in untreated *vs.* extracted blood samples drawn during cardiopulmonary bypass

patient number	untreated [µg/l]	after extraction [µg/l]	
1	0.5	0.5	
2	0.5	0.9	
3	0.4	0.3	
4	0.3	0.3	
5	0.1	0.1	
6	-0.1	0.4	
7	0.3	0.7	
8	1.2	1.6	
9	0.1	0.3	significance of difference (t test)
mean	0.4	0.6	$p < 0.05$
pump priming	-0.3 (± 0.1)	0.0	$p < 0.01$

The "in vitro" influence of Haemaccel is illustrated in figure 3, representing a normal calibration curve and a calibration curve with addition of Haemaccel to the standards at a concentration of 5 g/l. From figure 3 one may see that the curves a and b do not run parallel. This means that there is no constant effect of Haemaccel in the digoxin concentration range we studied. When antibody is omitted (i.e. the blank), more radioactivity is measured in the presence of Haemaccel than in its absence. Less [^{3}H]digoxin is adsorbed to charcoal, depending on the Haemaccel concentration. When blanks were measured at different known unlabelled digoxin concentrations, no decrease of this effect could be detected. In other words, the influence of Haemaccel on the binding of digoxin to charcoal is independent of the digoxin concentration. There are no signs of interference in beta counting caused by quenching or chemiluminescence. So the effect must be attributed to interference with the adsorption to charcoal and possibly also with the formation of the antibody-antigen complex.

Though a combined administration of digoxin and Haemaccel is very rare, under these circumstances the observed interference may give rise to a misleading interpretation of serum digoxin assay results, especially at high digoxin levels. At zero digoxin concentrations a decrease of 0.1 µg/l is found; at a known digoxin value of 3.0 µg/l, the measured value may be decreased by as much as 1.0 µg/l. This interference was not observed with two other plasma substitutes, Macrodex and Rheomacrocex.

[1]) The effects of Haemaccel and ethanol were studied with antibody charge K9100.
A recently delivered lot (K0061) gave similar results.

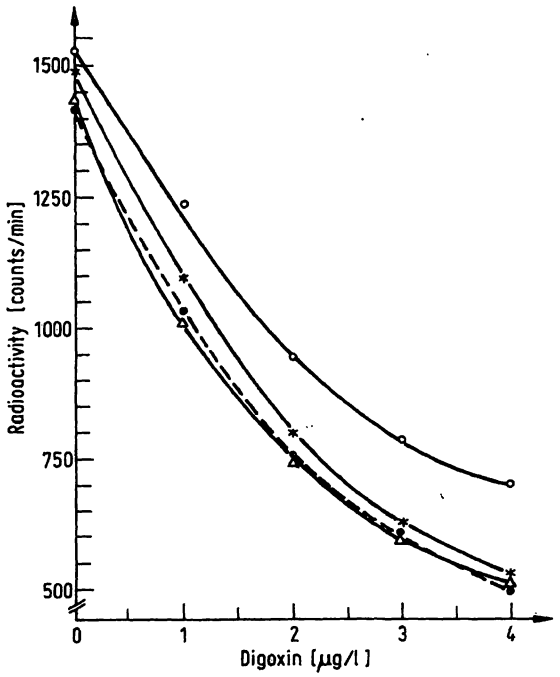

Fig. 3. Effect of Haemaccel (5 g/l) on the radioimmunoassay of digoxin.
curve a: ★——★: normal calibration curve
curve b: o——o: calibration curve in the presence of Haemaccel
curve c: ● — — ●: as curve a after extraction
curve d: △——△: as curve b after extraction
No correction was made for final volume differences (see text)
Each point represents the mean of four determinations. The coefficient of variation of cpm's was < 5%. Significance of difference between the points of curve a and b: $p < 0.02$ at 0 µg/l digoxin and $p < 0.01$ at all other concentrations. Curve c and d did not differ significantly.

To avoid the problems caused by Haemaccel, digoxin can be extracted from the samples with methylene chloride; as illustrated in figure 3, curves c and d, normal and Haemaccel-containing standards then give the same values. For a clearer presentation no correction was made for the fact that the final volume of 1.0 ml for extractions really represents 1.2 ml of sample.

It should be noticed that this extraction is not a standard procedure in the digoxin radioimmunoassay, and that the presence of Haemaccel in serum is not as easily recognized as haemolysis, jaundice or radioactive contamination.

Provided that Haemaccel does not interfere with the antibody-antigen binding, the observed phenomenon should be a stimulus to use separation techniques in which the antibody-antigen complex is removed, for example, by immuno-precipitation or by a solid phase antibody.

Ethanol[1])

From the differences between extracted and unextracted samples, mentioned above, at zero digoxin concentration and without Haemaccel, we concluded that ethanol might have an effect on the assay (see curves *a* and *c* in Fig. 3). To study this influence we replaced the normal buffer with buffer containing a quantity of ethanol equivalent to or twice that used after extraction.

The result of this addition is presented in figure 4. It is clear that ethanol causes a small positive error in the assay. Ten percent ethanol in a sample causes about 0.1–0.2 µg/l increase in the observed digoxin concentration ($p < 0.05$).

In patients undergoing cardiopulmonary bypass low plasma digoxin concentrations are to be expected, since digoxin administration is usually discontinued 48 hours prior to surgery. The small positive effect of ethanol may therefore lead to errors of up to 100% in the observed tissue: plasma ratio of digoxin. Moreover this experiment shows that the standards for the assay should not contain ethanol. Variations in results between commercially available kits may be caused by differences in the solvent of the standards.

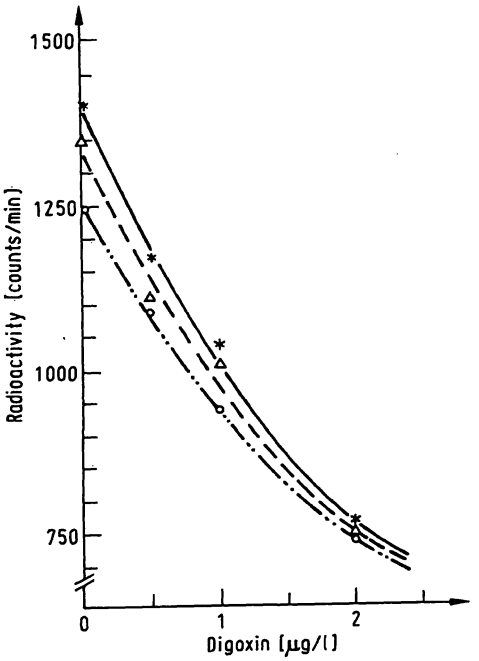

Fig. 4. Effect of ethanol on the radioimmunoassay of digoxin.
curve a: ★——★: normal calibration curve
curve b: △——△: calibration curve with ethanol (1/9 by vol.)
curve c: o···—···o: calibration curve with ethanol (1/4 by vol.)
Each point represents the mean of four determinations. The coefficient of variation of cpm's was < 5%. Significance of difference between the points of:

	curve a and b	curve a and c
0.0 µg/l	$p < 0.05$	$p < 0.01$
0.5 µg/l	N. S.	$p < 0.05$
1.0 µg/l	N. S.	$p < 0.05$
2.0 µg/l	N. S.	N. S.
The mean bias of all points:	40 cpm $p < 0.05$	93 cpm $p < 0.05$

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Drs. A. B. T. J. Boink
Department of Cardiology and Cardiovascular Surgery
University Hospital
Catharijnesingel 101
Utrecht, The Netherlands